

Nuclear Factor 1 Family Members Mediate Repression of the BK Virus Late Promoter

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BK virus (BKV) is a member of the polyoma virus family that is ubiquitous in humans. Its 5-kb DNA genome consists of a bidirectional promoter region situated between two temporally regulated coding regions. We mapped the transcription initiation site of the major late promoter (MLP) of the archetype strain BKV(WW) to nt 185. We found that it lies within the sequence TGGN₆GCCA, a binding site for members of the nuclear factor 1 (NF1) family of transcription factors. Competition electrophoretic mobility shift and immunoshift assays confirmed that NF1 factors present in nuclear extracts of HeLa and CV-1 cells bind to the BKV-MLP. Because BKV(WW) grew poorly in tissue culture and failed to express detectable levels of RNA *in vitro*, SV40-BKV chimeric viruses were constructed to investigate the transcriptional function of this NF-1 binding site. These sequence-specific factors repressed transcription in a cell-free system when template copy number was low. This repression could be relieved by the addition in *trans* of oligonucleotides containing wild-type, but not mutated, NF1-binding site sequences. SV40-BKV chimeric viruses defective in this NF1-binding site overproduced late RNA at early, but not late, times after transfection of CV-1 cells. Finally, transient expression in 293 cells of cDNAs encoding the family members NF1-A4, NF1-C2, and NF1-X2 specifically repressed transcription from the BKV late promoter approximately 3-, 10-, and 10-fold, respectively, in a DNA binding-dependent manner. We conclude that some members of the NF1 family of transcription factors can act as sequence-specific cellular repressors of the BKV-MLP. We propose that titration of these and other cellular repressors by viral genome amplification may be responsible in part for the replication-dependent component of the early-to-late switch in BKV gene expression. © 2001 Academic Press

INTRODUCTION

BK virus (BKV) is a member of the polyoma virus family of oncogenic DNA viruses (Gardner *et al.*, 1971; Sief *et al.*, 1979; Howley, 1980; Small *et al.*, 1986). Approximately 80% of humans have been infected with BKV by adulthood (Schreier and Gruber, 1986). The virus primarily infects the kidney, where it persists in a largely latent and undetectable state. However, immunosuppression of the host by pregnancy, disease, or treatment with certain drugs frequently leads to active replication of the virus (Schreier and Gruber, 1986).

Several different strains of BKV have been isolated and sequenced. All of them show extensive sequence homology in their two protein-coding regions, but considerable heterogeneity in their bidirectional promoter regions (Rubinstein *et al.*, 1989; Rubinstein, 1991; Negrini *et al.*, 1991). Whether these strains exist in nature or are an artifact of passage in cells in culture remains unclear (Rubinstein and Harley, 1989). One strain, BKV(WW), isolated and cloned directly from human urine, is considered to be the archetype strain (Chauhan *et al.*, 1984; Rubinstein *et al.*, 1987; Rubinstein and Harley, 1989; Negrini *et al.*, 1991).

As with simian virus 40 (SV40), a monkey homolog of

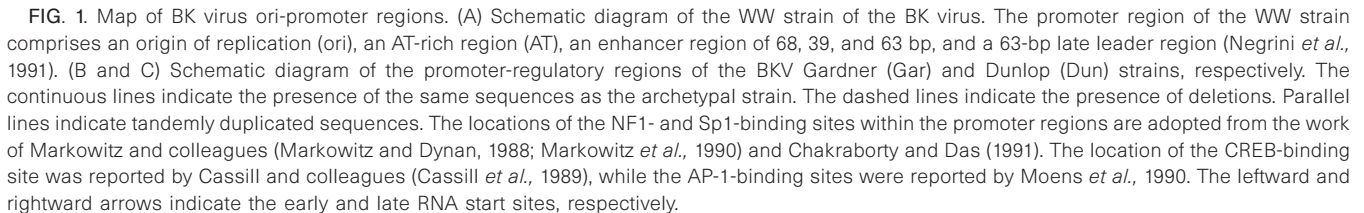
BKV, the early region of BKVs 5-kb genome is expressed early in the lytic cycle of infection. It encodes the large (T) and small tumor (t) antigens. Large T antigen autoregulates early gene transcription (Deyerle *et al.*, 1989) and plays multiple roles in viral DNA replication and transactivation of late gene expression (Cassill and Subramani, 1988; Cassill *et al.*, 1989). The late genes encode the virion proteins, VP1, VP2, and VP3, and a leader-encoded protein called agnoprotein. The late region is expressed at significant levels only after the onset of viral DNA replication.

Our laboratory has been studying the mechanisms by which the SV40 genes are temporally regulated. We have identified hormone response elements within the proximal region of the major late promoter of SV40 that specifically bind members of the hormone receptor superfamily. These factors repress late gene expression when template copy number is low (Wiley *et al.*, 1993; Zuo and Mertz, 1995; Zuo, 1995; Zuo *et al.*, 1997).

We have speculated that temporal regulation of other DNA tumor viruses might also occur in part by transcriptional repression of their late promoters. In the experiments presented here, we tested this hypothesis with BKV. We show that sequences surrounding the major transcription initiation site of the late promoter of BKV strain WW can sequence-specifically bind members of the NF1 family of transcription factors. As with SV40, the binding of these cellular factors results in repression of

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We chose to investigate the regulation of late gene expression of the archetype BKV strain WW (see WW strain, Fig. 1A), a strain isolated and cloned directly from human urine without passage in tissue culture. Since significant quantities of this BK virus strain appear in human urine under certain physiological conditions (Chauhan *et al.*, 1984), a productive viral life cycle must occur in its natural human host. Thus, BKV(WW) might be of greater biological relevance than strains preselected to grow well under artificial laboratory conditions. Unfortunately, when BKV(WW) is cultured in established human cell lines, neither viral early RNA nor viral DNA is produced in significant quantities (Markowitz and Dynan, 1988; Rubinstein and Harley, 1989). Our attempts to detect BKV late RNA by either S1 nuclease mapping or primer extension techniques in either human 293 cells or primate CV-1PD cells transfected with BKV(WW) DNA

Previously, Cassill and Subramani (1988) mapped the 5' ends of the late mRNAs synthesized from the Gardner strain of BKV. They identified a cluster of transcription start sites within the c element (see Gar strain, Fig. 1B). Together, these start sites account for approximately 70% of the late viral mRNAs accumulated in CV-1 cells. They

proved unsuccessful (data not shown). It is known that SV40 T-antigen binds to the T antigen-binding sites of BKV, supporting genomic replication (Ryder *et al.*, 1983). Nevertheless, we also failed to detect BKV late RNA in SV40 T antigen-containing COS-M6 cells transfected with BKV(WW) DNA (data not shown). Possibly, still missing was a cellular- or virus-specific function contained within T antigen's carboxyl-terminal domain needed for biogenesis of BKV late mRNA.

To overcome these obstacles, we constructed the plasmid pSVBK2 (Fig. 2A). This plasmid contains a chimeric SV40/BKV genome in which (i) the late promoter-regulatory region of SV40 has been replaced with the corresponding region of BKV(WW) and (ii) the retained initiator element of the SV40-major late promoter (MLP) has been inactivated. Thus, viral late RNA should primarily be synthesized starting from sequences within the BKV promoter region. This chimeric genome retains SV40's early promoter, large T antigen-encoding region, origin of viral DNA replication, and late RNA processing signals and protein-coding regions. Therefore, the viral DNA should replicate to high copy number in African green monkey kidney cells, with its late transcripts being efficiently processed such that their 5' ends could be readily mapped.

We transfected primate COS-M6 cells with SVBK2 DNA, harvested whole-cell RNA 48 h later, and analyzed the resulting viral RNAs by primer extension. High levels of BKV-initiated late RNA were detected, with the 5' ends mapping predominantly to two sites (Fig. 2B, lane 2). The major 5' end accounted for approximately 45% of the total viral late RNA and mapped to BKV nt 185 in the BKV numbering system of Negrini *et al.* (1991). The second site accounted for approximately 30% and mapped to BKV nt 282. This latter site lies downstream of the first ATG codon at BKV nt 266. As a control, we mapped RNA harvested from COS-M6 cells transfected with XS13, a mutant of SV40 that lacks the enhancer region (Fromm and Berg, 1982); we were unable to detect any BKV RNA (Fig. 2B, lane 3). Thus, it is unlikely that the RNA synthesized from SVBK2 DNA arose from transcription within SV40 sequences.

To determine more accurately the major site of transcription initiation from BKV(WW)'s late promoter, these primer extension products were also electrophoresed in a high-resolution gel alongside a sequencing ladder of pSVBK2 DNA. The 5' end of the SV40/BKV chimeric major late RNA mapped predominantly to two adjacent bases within the BKV promoter region, nts 185 and 186 (Fig. 2C, lane 2; summarized in Fig. 2D). Although these 5' ends were identified within the context of an SV40 replicon, we conclude that BKV nts 185 and 186 are probably the major sites of transcription initiation from the BKV(WW) late promoter.

We previously reported a method involving weight matrices that can be used to predict the relative strengths of

given sequences as functional Inrs and TBP-binding sites of promoters transcribed by RNA polymerase II (Kraus *et al.*, 1996). Using our Inr weight matrix, we calculated that the sequence surrounding the BKV(WW)-MLP start site mapped here, 5'-CCAAAG-3', likely functions as a moderately good Inr, with a value approximately one-fifth that of the optimal initiator sequence 5'-NNCA(G/T)T-3'. Situated 24–30 bp upstream of this transcription initiation site is the sequence 5'-AATAAAC-3' (Fig. 2D). Using our TBP-binding site weight matrix (Kraus *et al.*, 1996), we calculated that this sequence probably binds TBP quite well, functioning as the –30 region element of an RNA polymerase II promoter with a value approximately two-thirds that of an optimal TATA box. We conclude that the late promoter of BKV strain WW identified here possesses fairly strong basal promoter elements. Thus, it is also likely the major late promoter of this virus in its natural context and physiological state even though we were unable to reach this conclusion directly. For the purpose of interpreting the experiments described below, we will assume this conclusion is valid.

NF1 family members bind the BKV(WW)-MLP

It has been shown previously that members of the steroid/thyroid/retinoid hormone receptor superfamily bind directly at the initiation site of SV40's major late promoter (+1 site) and 55 bp downstream of it (+55 site), repressing transcription of the promoter when template copy number is low (Wiley *et al.*, 1993). These nuclear receptors include estrogen-related receptor α 1 (Wiley *et al.*, 1993, Johnston *et al.*, 1997), thyroid receptor α 1 as a heterodimer with retinoid X receptor (Zuo, 1995; Zuo *et al.*, 1997), testis receptor 2 (Zuo, 1995), and the human homolog of chicken ovalbumin upstream promoter transcription factor 1 (Zuo and Mertz, 1995; Zuo, 1995).

To examine whether the initiator region (Inr) of the BKV(WW)-MLP might also bind one or more of the nuclear receptors that recognize the Inr of the SV40-MLP, we performed competition electrophoretic mobility shift assays (EMSAs) with these nuclear receptor proteins and radiolabeled double-stranded oligonucleotides corresponding to the SV40-MLP +1 and +55 sites as probes. A 30-bp double-stranded oligonucleotide corresponding to BKV(WW) nt –17 to nt +13 relative to its major late initiation site failed to compete for binding to these receptor proteins (data not shown). This BKV-MLP DNA sequence also failed to compete with these SV40-MLP DNA sequences for binding other proteins present in a HeLa cell nuclear extract (data not shown). Therefore, despite the high degree of relatedness between SV40 and BKV in their protein-coding regions, the major late promoters of these two viruses are not bound by the same nongeneral factors.

To look for other factors that might be involved in

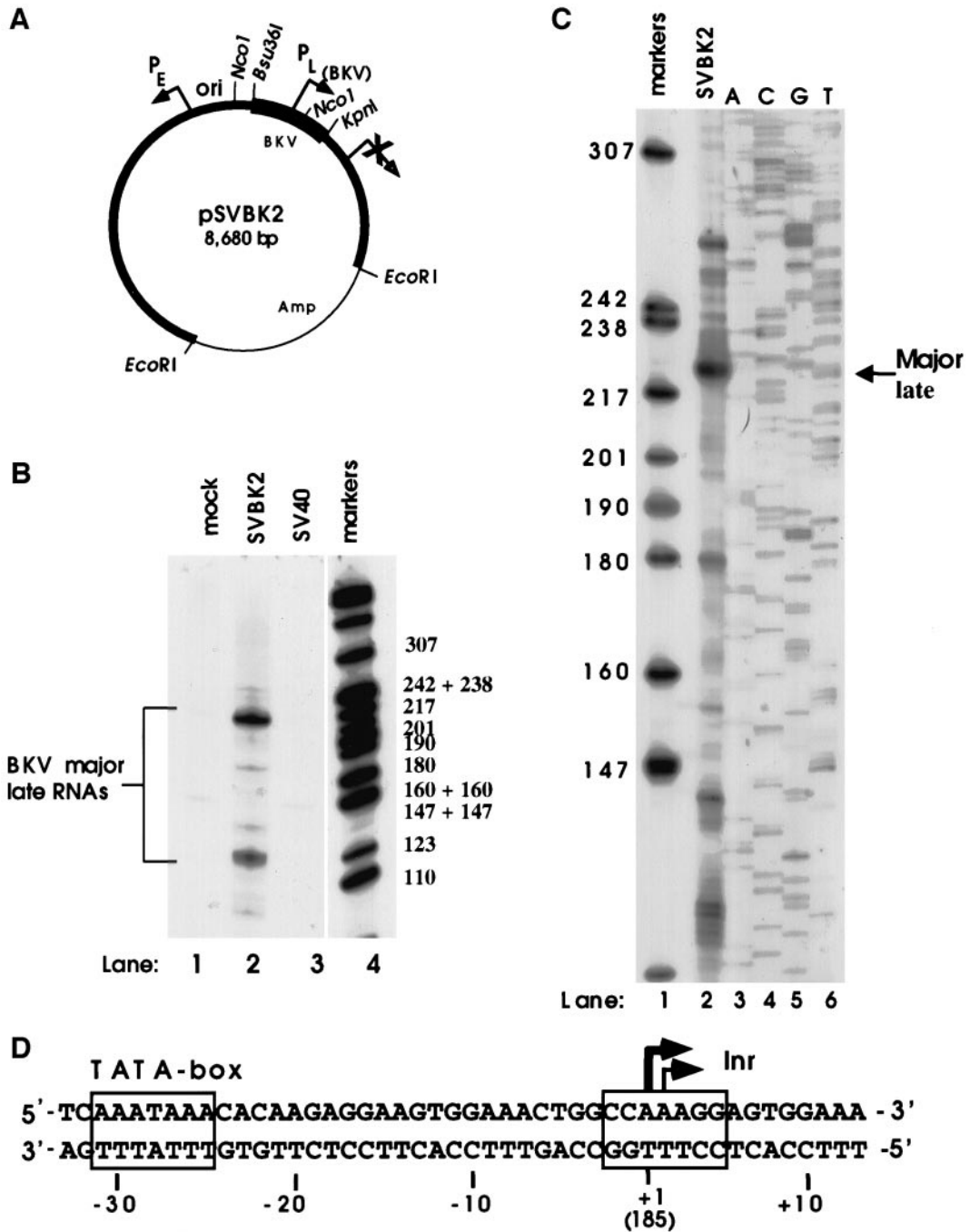


FIG. 2. Identification of BKV's major late promoter. (A) Schematic representation of the SV40-BKV chimeric plasmid used to map the transcriptional start site of the BKV-MLP. See Materials and Methods for details of the plasmid construction. Thin line, bacterial cloning vector sequence; thick black line, BKV sequence extending from the *Bsu36I* to the *KpnI* restriction sites; thick gray lines, SV40 sequences; P_E and P_L, early and late promoters, respectively, indicating the directions of transcription. (B) Primer extension analysis of the 5' ends of the late viral RNAs accumulated in COS-M6 cells 48 h after transfection with 3 μ g/10-mm dish of the indicated DNAs. All RNAs were mapped using a primer corresponding to BKV nts 227–203. Lane 1, sample from mock-transfected cells; lane 2, cells transfected with the chimeric construct SVBK2; lane 3, transfected with SV40 mutant, XS13; lane 4, *MspI*-cut pBR322 DNA. (C) Localization of the 5' ends of the RNAs synthesized from the BKV late promoter. Whole-cell RNA was harvested from COS-M6 cells transfected 48 h earlier with SVBK2 DNA and analyzed by primer extension with the same BKV late RNA-specific primer used in B. Shown here is an autoradiogram of the resulting products electrophoresed in a 7 M urea, 6% polyacrylamide gel (lane 2) alongside dideoxy sequencing reactions generated with the same primer (lanes 3–6). Lane 1, *MspI*-cut pBR322 DNA. (D) Schematic representation of the sequences surrounding BKV's major late promoter. Arrows indicate the location(s) of the major late transcription initiation site(s) mapped in C. The boxes enclose the sequences that comprise the putative initiator (Inr) and TBP-binding (TATA box) site elements of this promoter. The nucleotide residues are numbered relative to the most prominent initiation site as determined from the data in C; +1 corresponds to nt 185 in the numbering system of BKV(WW) as described by Negrini *et al.* (1991).

regulating transcription from the BKV-MLP, we performed direct EMSAs using nuclear extracts from HeLa, CV-1, and 293 cells as protein sources and a radiolabeled double-stranded oligonucleotide corresponding to the 30-bp region of the BKV(WW)-MLP described above as probe (Fig. 3A). Multiple DNA-protein complexes were observed using HeLa cell nuclear extract (Fig. 3A, lanes 2 and 3), while two relatively fast-migrating species were observed in the CV-1 nuclear extract (Fig. 3A, lanes 4 and 5). Interestingly, the human kidney cell line 293, derived from the host tissue and known to enable production of BK virus, appeared to be devoid of factors that bound the sequences within this region of the BKV-MLP (Fig. 3A, lanes 6 and 7).

Markowitz and Dynan (1988) found sites within the promoter region of the BKV(WW) strain that were protected from DNaseI cleavage by incubation with a partially purified protein preparation obtained from HeLa cell nuclei. Five of these sites, including one overlapping the Inr of the BKV-MLP identified here, closely resemble the sequence TGGN₆GCCAA, the consensus binding site sequence for members of the NF1 family of transcription factors (Nagata *et al.*, 1982; Gronostajski *et al.*, 1985; Rosenfeld and Kelley, 1986) (Fig. 1A).

To test further whether the sequences surrounding the Inr of the BKV-MLP can, indeed, be recognized by NF1-like factors, we performed a series of competition electrophoretic mobility shift assays (Fig. 3B; see Fig. 3D for sequences of the oligonucleotides). As expected, the double-stranded oligonucleotide containing a known NF1-binding site competed as well as unlabeled BKV(WT) Inr DNA for binding the factors present in the HeLa cell nuclear extract (Fig. 3B, lanes 7 and 8 vs lanes 3 and 4). On the other hand, the double-stranded oligonucleotide BKV(mut), which differs from the oligonucleotide BKV(WT) solely in two base pairs known to be important for the binding of NF1 family members, failed to compete for binding any of these factors (Fig. 3B, lanes 5 and 6 vs lane 2). Therefore, these protein-BKV(WW) DNA complexes likely contain NF1 family members.

To confirm the identity of the proteins present in these complexes, we performed immunoshift assays using an antiserum made against the DNA-binding domain of NF1 that recognizes an epitope common to all members of the NF1 family (Fig. 3C). Whereas incubation with preimmune serum had no effect on the formation of any DNA-protein complexes (Fig. 3C, lane 3 vs lane 2), incubation with the NF1-specific serum resulted in both the appearance of a slower mobility complex and the marked decrease in the formation of the complexes C1, C3, and C4 (Fig. 3C, lane 4). Thus, we conclude that members of the NF1 family are the factors present in HeLa cells that bind sequence-specifically to the initiation site of the BKV(WW)-MLP.

NF1 family members repress transcription from the BKV(WW)-MLP at low template copy number

To examine the effects on transcription *in vivo* of binding of NF1 family members to the initiator region of the BKV(WW)-MLP, we first constructed the SV40-BKV chimeric plasmid pSVBK3(WT). This plasmid contains essentially a wild-type SV40 genome except that the sequences surrounding the initiator element of the SV40-MLP have been replaced with ones surrounding the initiator element of the BKV(WW)-MLP (see Fig. 4A for sequence). Thus, the viral late promoter is no longer regulated in part by the hormone receptor-binding site that surrounds the Inr of the SV40-MLP. Rather, we can test with this chimeric genome whether the viral late promoter can be regulated, instead, by the binding of NF1 family members to the NF1 binding site surrounding the Inr of the BKV(WW)-MLP. If the binding of NF1 family members to the BKV(WW)-MLP can regulate late transcription in a manner analogous to hormone receptor superfamily members binding the SV0-MLP, a mutation in the NF1 binding site that inactivates binding of the NF1 family members would be predicted to lead to the overproduction of viral late RNA at early times in the lytic cycle when viral template copy number is low. Conversely, by late times in the lytic cycle when template copy number is high due to viral genome replication, the effect of this mutation on the accumulation of viral late RNA would be significantly reduced because titration would have eliminated in large part promoter occupancy by the cellular repressors on the wild-type template as well. Thus, we also constructed plasmid pSVBK3(mut), a variant of pSVBK3(WT) in which we had mutated the two base pairs crucial for the binding of NF1 family members (Fig. 4A).

To check that these chimeric SV40/BKV(WW)-MLPs function properly in transcription, the viral genomes containing them were excised from their cloning vectors and transfected in parallel into CV-1PD cells. The 5' ends of the viral late RNAs accumulated by 48 h posttransfection, a late time in the lytic cycle of infection of monkey cells, were determined by primer extension analysis (Fig. 4B). As expected, predominantly one species of late RNA was synthesized from the wild-type SV40-MLP (lane 4). In the SVBK3(WT)-transfected cells, transcripts originated at high levels from the previously mapped initiation site (Fig. 2C vs Fig. 4B, lane 2). However, they also originated at high levels from a new, slightly upstream site positioned at a more appropriate distance from the previously identified weak TATA box of the SV40-MLP (Wiley *et al.*, 1992) and at numerous weakly used sites. Essentially identical results were obtained with SVBK3(mut), except for a one-base shift in the major site of initiation likely caused by the alteration in the specific sequence of the initiator element (Fig. 4B, lane 3 vs lane 2). Also noteworthy is the lack of a significant effect of the mutation

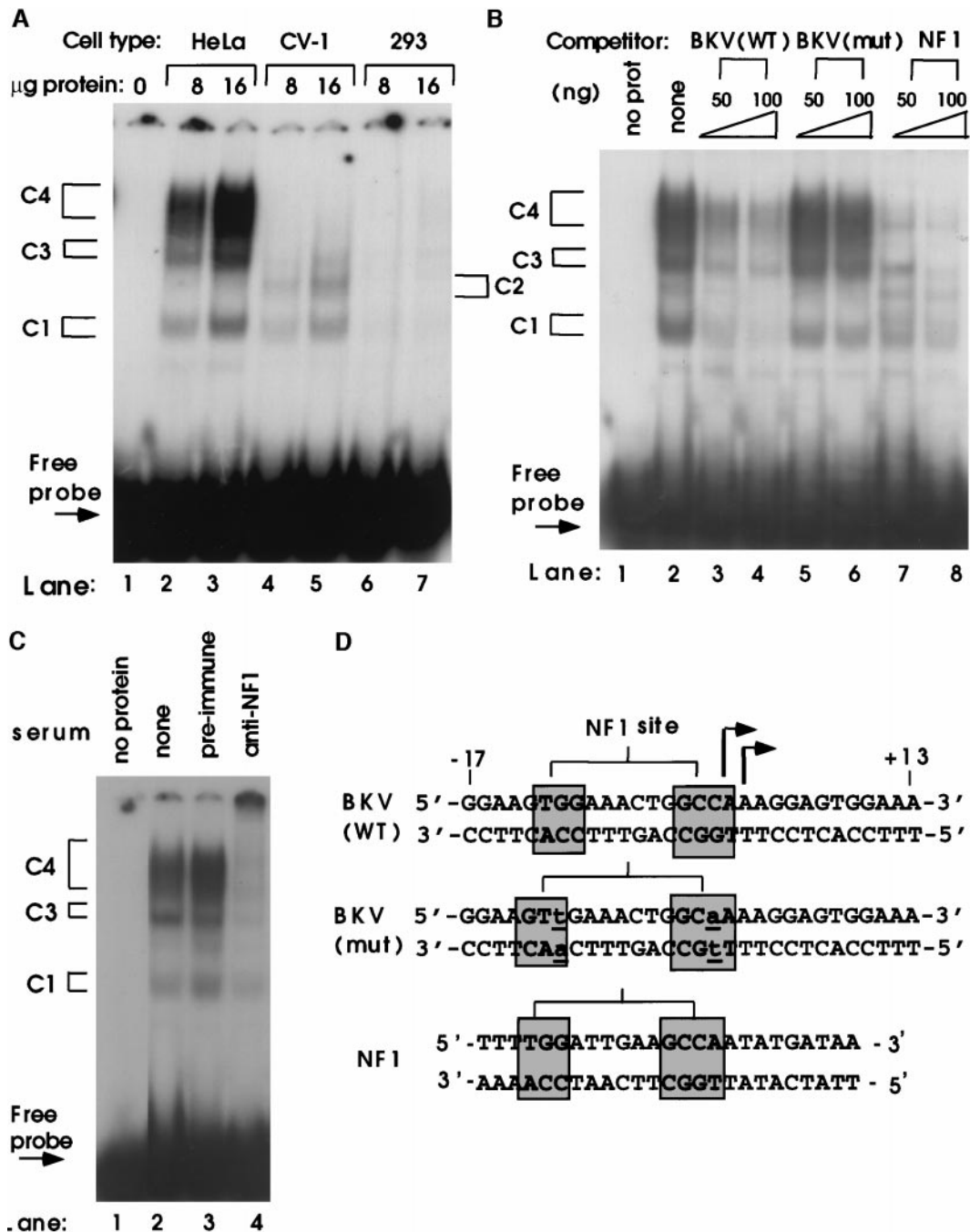


FIG. 3. NF1-like factors bind the initiator of the BKV-MLP. (A) Cell type-specific binding to the initiation site region of the BKV-MLP by factors present in HeLa, CV-1, and 293 cell nuclear extracts. Electrophoretic mobility shift assays (16 μ l per reaction) were performed by incubation of the indicated amounts of HeLa (lanes 2 and 3), CV-1 (lanes 4 and 5), and 293 (lanes 6 and 7) cell nuclear extracts with radiolabeled BKV(WT)-MLP DNA (0.5–1.0 ng) (see D for sequence) and electrophoresis in a native 4% polyacrylamide gel. (B) Sequence-specific competition for binding by oligonucleotides containing NF1-binding sites. Competition electrophoretic mobility shift assays were performed with HeLa cell nuclear extract, radiolabeled BKV(WT)-MLP DNA as probe, and the indicated DNAs as unlabeled competitors. The sequences of the DNAs are indicated in D; the NF1-binding site oligonucleotide corresponds to the one present in the adenovirus origin of DNA replication used in the original identification of NF1 (Nagata *et al.*, 1982). (C) Effects of anti-CTF/NF1 serum on formation and electrophoretic mobility of BKV(WT)-MLP DNA–protein complexes. EMSAs were performed with radiolabeled BKV(WT)-MLP DNA as probe, 10 μ g HeLa cell nuclear extract as protein source, and 1 μ l of the indicated serum. Lane 1, no protein; lane 2, no serum; lane 3, preincubation of the nuclear extract with a 1:10 dilution of preimmune serum; and lane 4, preincubation with a 1:100 dilution of an anti-CTF/NF1 serum (kindly provided by Dr. Noko Tanese). (D) Schematic representation of the sequences used as probe and competitor oligonucleotides in the experiments shown in A–C. The connected shaded boxes indicate the sequences that make up the two halves of the binding sites for members of the NF1 family of proteins.

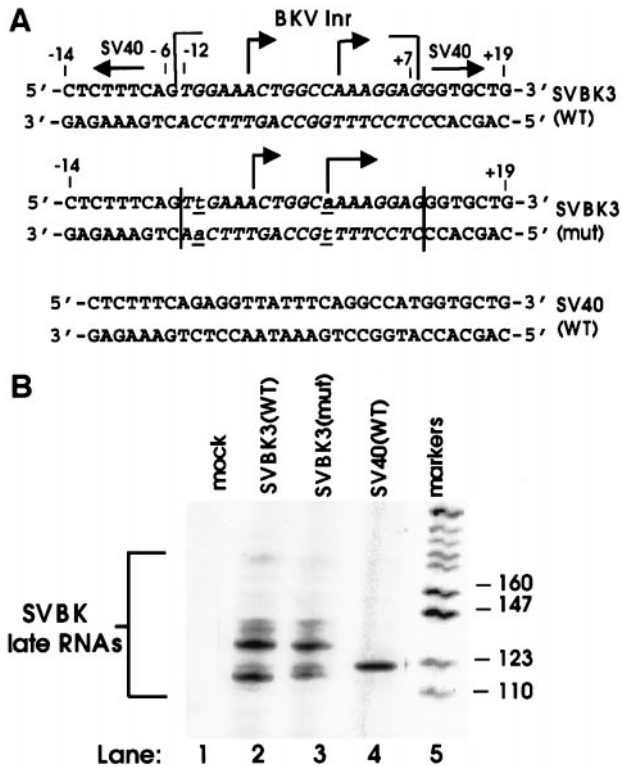


FIG. 4. Transcription initiation from the BKV-MLP Inr present within a chimeric SV40/BKV late promoter. (A) Schematic diagram indicating the sequences surrounding the initiation sites of the major late promoters in wild-type SV40, the chimeric SVBK3(WT), and a mutant variant of it, SVBK3mut. The arrows indicate the major sites of transcription initiation from these promoters as determined from the data presented in B. The sequences derived from BKV are indicated in italics; the bases altered in the mutant variant are underlined and lowercase. The nucleotide numbers are given relative to the predominant sites of initiation of the wild-type nonchimeric promoters. (B) Products of primer extension analysis of the 5' ends of the late viral RNAs accumulated in CV-1PD cells by 48 h after transfection with 3 μ g/10-mm dish of the indicated viral DNAs. The primer used corresponded to SV40 nt 394–369. Lane 1, sample from mock-transfected cells processed in parallel; lanes 2–4, samples from cells transfected with SVBK3, SVBK3mut, and WT SV40 DNA, respectively; and lane 5, *MspI*-cut pBR322 DNA. Lane 5 was exposed for a slightly longer period of time than lanes 1–4.

on the amount of viral late RNA accumulated (Fig. 4B, lane 3 vs lane 2). This latter finding is consistent with our hypothesis since the high-viral template copy number present by 48 h after transfection would be expected to titrate out putative sequence-specific repressors (Wiley *et al.*, 1993). Thus, we conclude that these chimeric promoters synthesize viral late RNA reasonably well.

To examine whether the binding of NF1 family members to the Inr of the major late promoter affects the synthesis of viral late RNA at earlier times in the lytic cycle of infection, we transfected CV-1PD cells as described above, but harvested the cells at earlier times posttransfection as well. The relative amounts of the various viral early and late RNA species accumulated in these cells were determined by quantitative S1 nuclease mapping (Fig. 5). As predicted, the results obtained were

fairly similar to ones observed previously with an SV40 mutant defective in the binding of hormone receptor superfamily members to the hormone response elements (HRE) that surround the Inr of the SV40-MLP (Wiley *et al.*, 1993). Cells transfected with SVBK3(mut) DNA accumulated four- to eightfold more major late-initiated RNA by 22 to 26 h posttransfection than did cells transfected in parallel with SVBK3(WT) DNA (Fig. 5A, lanes 9 and 10 vs lanes 3 and 4). This difference was down to twofold by 42 h posttransfection (Fig. 5A, lane 11 vs lane 5). Southern blot analysis showed, as expected, that the time course of accumulation of viral DNA was unaffected by the mutation in the NF1-binding site of the BKV-MLP (Fig. 5B). Therefore, inactivation of an NF1-binding site surrounding the Inr partially relieves repression of the BKV major late promoter at early times when template copy number is low; however, at late times in the lytic cycle when template copy number is high, the effect of the mutation becomes smaller, presumably because of titration of the NF1 family members that repress transcription of the wild-type promoter.

An alternative hypothesis consistent with these data is that the mutation in the initiator element creates a better basal promoter that is more transcriptionally active, with the loss of binding of NF1-like factors being coincidental rather than causative. If this were the case, removal of the NF1 family members by titration *in trans* would be predicted to have little, if any, effect on transcription. Thus, the NF1 binding site mutant would continue to overproduce late RNA relative to the wild-type promoter even when NF1 family members were no longer available to bind to the wild-type promoter.

To test this possibility, we examined the effects of various competitor oligonucleotides on transcription of pSVBK3X(WT) vs pSVBK3X(mut) in a cell-free transcription system derived from HeLa cells (Fig. 6). These viral DNA templates are identical to the ones used in the transfection experiment (see Fig. 4) except for the deletion of the 72-bp repeat enhancer region of SV40. Thus, transcription of the late promoter is efficiently activated by SV40's Sp1 binding sites (Fromm and Berg, 1982; Wiley *et al.*, 1993; Kraus *et al.*, 1996).

The addition *in trans* of 25 ng (1.25 pmol) and 50 ng (2.5 pmol) of double-stranded oligonucleotide containing the initiator region of the BKV-MLP resulted in a three- and sixfold increase, respectively, in transcription from the wild-type BKV-MLP (Fig. 6, lanes 3 and 4 vs lane 2), almost to the level of RNA synthesized from the mutant template (Fig. 6, lanes 7 and 8 vs lane 4). On the other hand, the addition *in trans* of similar quantities of double-stranded oligonucleotide containing the 2-bp mutation in the NF1-binding site had little, if any, effect on transcription from the wild-type promoter (Fig. 6, lanes 5 and 6 vs lane 2). As an additional control, the effects of the wild-type competitor oligonucleotide on transcription from the NF1-binding site mutant variant, pSVBK3X(mut), were ex-

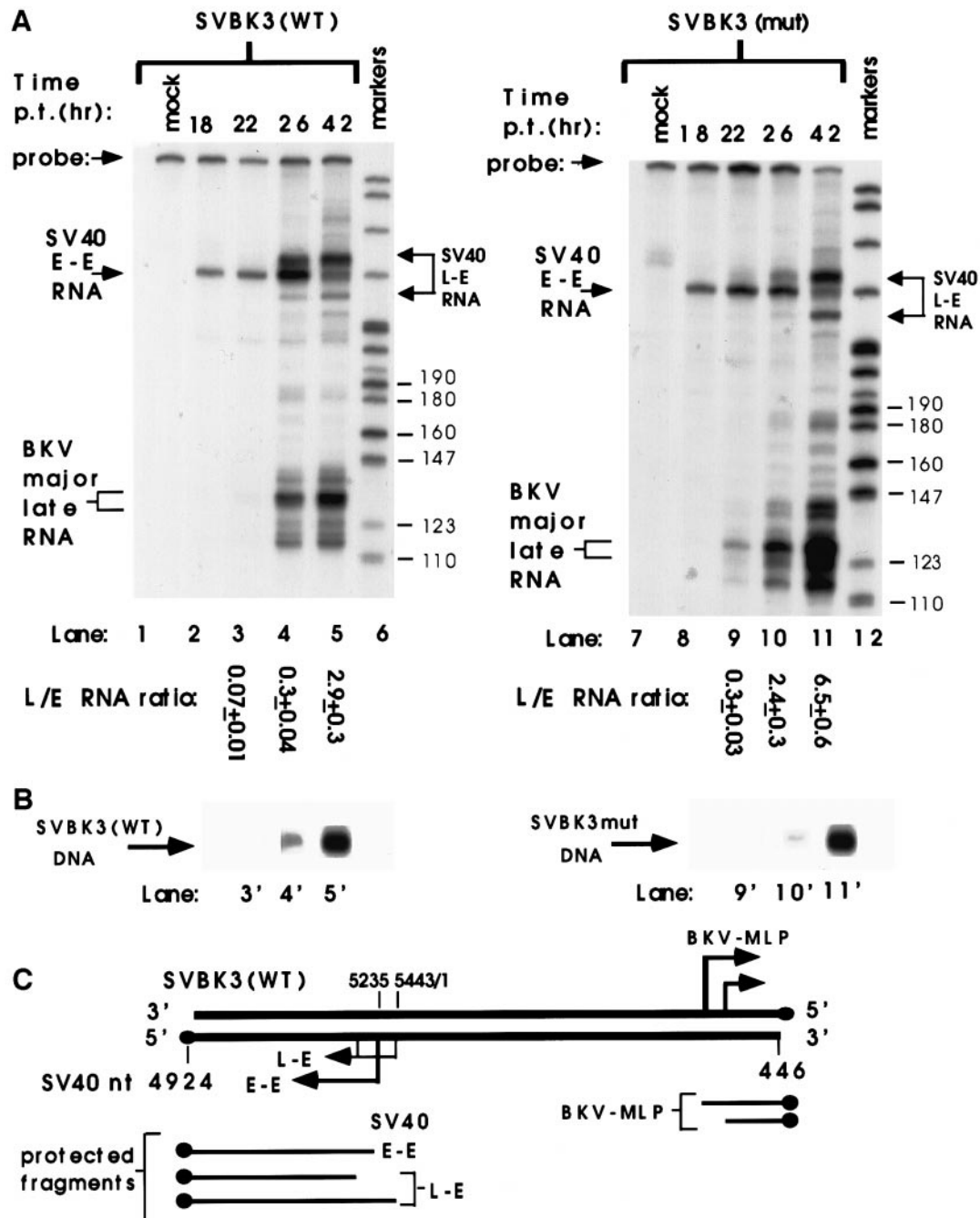


FIG. 5. Binding of NF1-like factors to the BKV major late promoter specifically represses BKV late RNA synthesis *in vivo*. (A) CV-1PD cells were transfected in parallel with 3 μ g/10-mm dish of SVBK3(WT) or SVBK3mut and incubated at 37°C for the times indicated. The relative amounts of the early and late viral RNAs present in the cells were analyzed by quantitative S1 nuclease mapping with the double-stranded probe depicted in C. Shown here is the resulting autoradiogram in which the S1 nuclease reaction mixtures contained 25% (lanes 1–3 and 7–9), 12.5% (lanes 4 and 10), and 5% (lanes 5 and 11) of the whole-cell RNA present in the dishes of cells. The bands indicated within the bracket labeled BKV major late RNA were used to quantify the amounts of BKV late RNA, while the bands indicated by the arrow SV40 E-E were used to quantify the amounts of SV40 early RNA. The numbers at the bottom indicate the amounts of BKV major late RNA relative to SV40 early–early RNA present at the indicated times after transfection. These numbers are means \pm SEM of data obtained from three independent experiments similar to the one shown here. The amounts of BKV major late RNA at 18 h were too low to quantify by this assay. (B) Southern blot analysis of the replicated chimeric viral DNAs present in the 22-, 26-, and 42-h samples analyzed in parallel for viral RNA in A. Each lane contained 2% (lanes 3', 4', 9', 10') or 1% (lanes 5' and 11') of the total DNA present in the sample processed as previously described (Hertz and Mertz, 1986). Lanes 3', 4', 9', and 10' were exposed twice as long as lanes 5' and 11'. (C) Structures of the probes used in the S1 nuclease mapping experiment shown in A and the fragments protected by the indicated RNAs. The numbers indicate the SV40 nucleotide residues in the SV40 numbering system of Buchman *et al.* (1982).

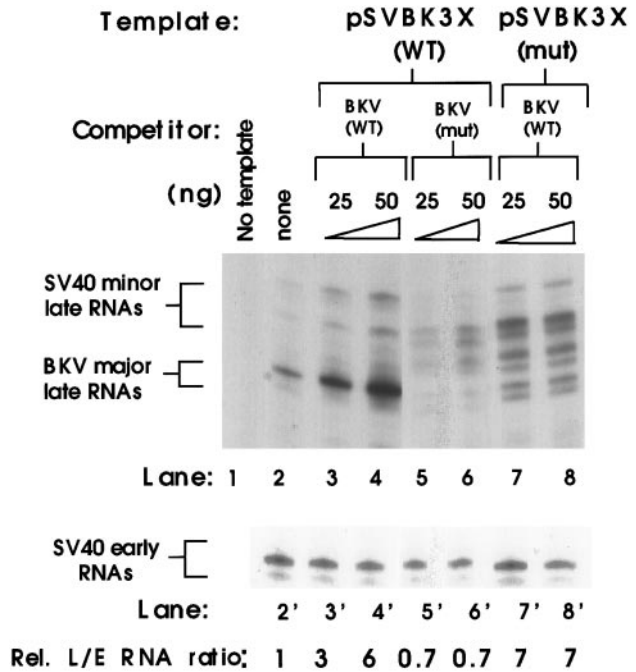


FIG. 6. Addition in *trans* of a competitor oligonucleotide containing an NF1-binding site specifically relieves repression of BKV late RNA synthesis in a cell-free transcription system. A HeLa cell nuclear extract was preincubated at 26°C for 15 min with 25 or 50 ng of a double-stranded oligonucleotide corresponding to the BKV-MLP NF1-binding site (lanes 3, 4, 7 and 8) or a mutant variant of it (lanes 5 and 6) (see Fig. 3D for sequences). Afterward, 200 ng supercoiled plasmid DNA of the indicated wild-type (lanes 2–6) or NF1-binding site mutant (lanes 7 and 8) was added as template and transcription was started by the addition of NTPs. The viral early and late RNAs synthesized from these templates were analyzed by primer extension with primers corresponding to SV40 nt 5136–5160 and 446–422, respectively. The numbers at the bottom indicate the ratios of BKV major late to SV40 early RNAs synthesized in each reaction mixture normalized to the late-to-early ratio obtained with pSVBK3X(WT) in the absence of competitor oligonucleotide (lane 2).

amined in parallel. As expected, the mutant promoter was transcribed at high levels regardless of the amount of competitor present in the reaction (Fig. 6, lanes 7 and 8). Also noteworthy is the absence of effects of these competitor oligonucleotides on transcription from the SV40 early promoter present on the same templates (Fig. 6, lanes 1'–8'). Thus, the effects of these oligonucleotides are sequence specific. We conclude that NF1 family members can act as sequence-specific repressors of the BKV(WW)-MLP.

Finally, we investigated the effects of the wild-type competitor oligonucleotide on transcription of pBKV(WW) and pSVBK2 in the cell-free transcription system. Regardless of the conditions tried, we failed to detect late RNA (data not shown). However, we have successfully investigated the regulation of the late promoter of the BKV(WW) strain in a nonchimeric context by using luciferase reporter constructs (see below).

NF1-A4, NF1-C2, and NF1-X2 sequence-specifically bind sequences overlapping the Inr region of the BKV(WW)-MLP

We next examined specific members of the NF1 family for binding to the NF1 site overlapping the initiator region of BKV(WW)-MLP. NF1-A4 and NF1-C2, members of the NF1-A and NF1-C subfamilies, respectively, share common amino termini and DNA-binding domains, but differ in their carboxyl termini. NF1-X2, a member of the NF1-X subfamily, differs in both the amino and the carboxy domains from NF1-A4 and NF1-C2 (Kruse and Sippel, 1994). The protein sources for these assays were whole-cell extracts prepared from 293 cells that had been transfected 2 days earlier with the expression plasmids pCHNF1-A, pCHNF1-C, and pCHNF1-X that encode NF1-A4, NF1-C2, and NF1-X2, respectively. Since 293 cells lack significant amounts of factors that bind the NF1 site of the BKV(WW)-MLP (Fig. 3A), the binding factors present in these extracts should have been largely synthesized from the expression plasmids. As expected, these extracts generated plasmid-specific protein–DNA complexes that exhibited different migration rates and binding patterns (Fig. 7). Binding of NF1-A4 to the Inr region of the BKV(WW)-MLP generated a single, fast-migrating protein–DNA complex (Fig. 7, lane 2), while the binding of NF1-C2 and NF1-X2 generated slower migrating multicomponent complexes (Fig. 7, lanes 5 and 8, respectively). These complexes are, presumably, the re-

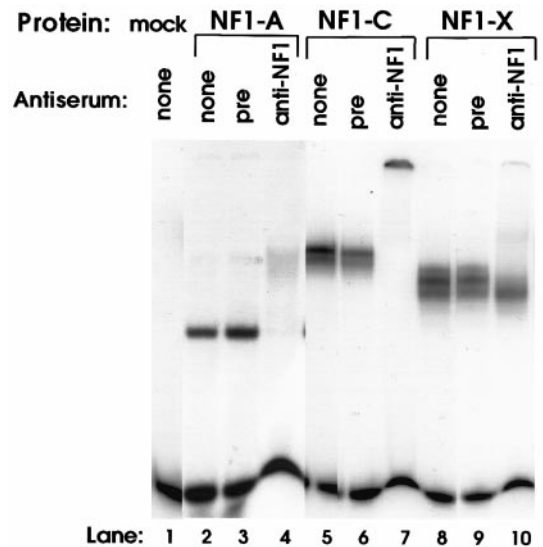


FIG. 7. NF1-A4, NF1-C2, and NF1-X2 bind the initiator of the BKV-MLP. EMSAs were performed with radiolabeled BKV(WT)-MLP DNA as probe, the indicated serum, and the whole-cell extracts obtained from 293 cells (approximately 5 μ g of protein) containing overexpressed NF1-A4 (lanes 2–4), NF1-C2 (lanes 5–7), and NF1-X2 (lanes 8–10) as protein sources. Lane 1, no protein or antiserum; lanes 2, 5, and 8, overexpressed NF1-A4, NF1-C2, and NF1-X2 alone, respectively; lanes 3, 6, and 9, preincubated with a 1:10 dilution of preimmune serum; and lanes 4, 7, and 10, preincubated with a 1:100 dilution of an anti-CTF/NF1 serum.

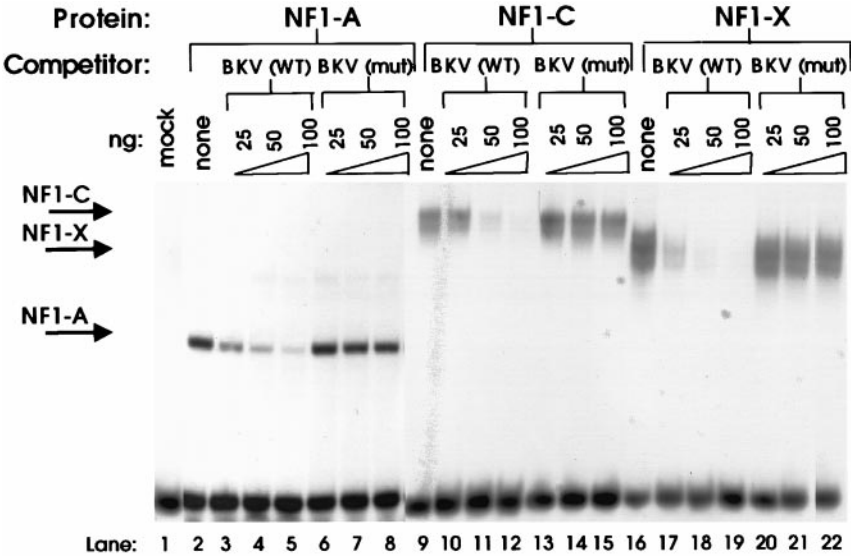


FIG. 8. Binding of NF1-A4, NF1-C2, and NF1-X2 to the *Inr* of the BKV-MLP is sequence specific. Competition EMSAs were performed with radiolabeled BKV(WT)-MLP DNA as probe, oligonucleotides containing the BKV-MLP(WT), or BKV-MLP(mut) sequences shown in Fig. 3D as competitors, and the indicated whole-cell extract obtained from 293 cells (approximately 5 μ g of protein) containing overexpressed NF1 factors as protein source.

sult of NF1 homomeric and/or heteromeric protein–protein interactions. The NF1-A4 and NF1-C2 protein–DNA complexes were lost or shifted in mobility following incubation with an antiserum made against the common DNA-binding domain of NF1 proteins (Fig. 7, lane 4 vs 2 and lane 7 vs 5, respectively), indicating that these specific NF1 family members bind to the *Inr* of the BKV(WW)-MLP. Interestingly, not all of the bands that comprise the NF1-X2 protein–DNA complex were lost or immuno-shifted following incubation with the anti-NF1 antiserum (Fig. 7, lane 10 vs 8). Thus, NF1-X2 can form a specific protein–DNA complex that apparently blocks the epitope on NF1-X2 recognized by this antibody.

Competition EMSAs showed that each of the NF1 complexes formed on the *Inr* of the BKV-MLP bound sequence-specifically (Fig. 8), including the NF1-X2 antibody-resistant complex (Fig. 8, lanes 16–19). Thus, we conclude that some members of the NF1 family of proteins bind efficiently and sequence-specifically to the *Inr* of the BKV(WW)-MLP. Indeed, it is quite likely that most, if not all, NF1 family members can bind the *Inr* of the BKV(WW)-MLP.

Overexpression of NF1-A4, NF1-C2, and NF1-X2 represses transcription of the BKV(WW)-MLP in 293 cells

Last, we examined the effect of overexpression of specific NF1 family members on expression of the BKV(WW)-MLP. A plasmid, pBKLuc (see Fig. 9A), in which the BKV(WW)-MLP drives expression of a luciferase reporter, was cotransfected into 293 cells along with plasmids expressing each of the specific NF1 family

members studied above. As controls, luciferase reporters driven by the SV40 early promoter (pGL3, Promega Corp., Madison, WI) and the BZLF1 promoter of EBV (pWTZpLUC, Kraus *et al.*, 2001), both of which lack any known NF1 binding sites, were cotransfected in parallel into 293 cells along with the NF1 expression plasmids. We found that overexpression of these NF1 family members led to repression of transcription from the BKV late promoter. NF1-C2 and NF1-X2 repressed transcription from the BKV late promoter approximately 10-fold, while NF1-A4 repressed approximately threefold (Fig. 9B). As expected, overexpression of these NF1 family members had little effect on transcription from the EBV BZLF1 and SV40 early promoters (Fig. 9B). Thus, squelching of general transcription factors or cofactors by the NF1 factors can be ruled out. Taken together, these findings show that specific members of the NF1 family can repress transcription of the BKV(WW) late promoter in a sequence-specific manner. They also show that NF1 factors can repress transcription within the context of the BKV late promoter, not only in the context of an SV40 chimera.

DISCUSSION

The experiments presented here were designed to test whether repression of the late genes of BKV contributes to the temporal regulation of this virus. We chose the human archetype strain, BKV(WW), since it is the strain naturally present in the human population. Unfortunately, we were unable to detect viral late mRNA in any of the cell types we transfected with BKV(WW) DNA (data not shown). These results are in agreement with those of

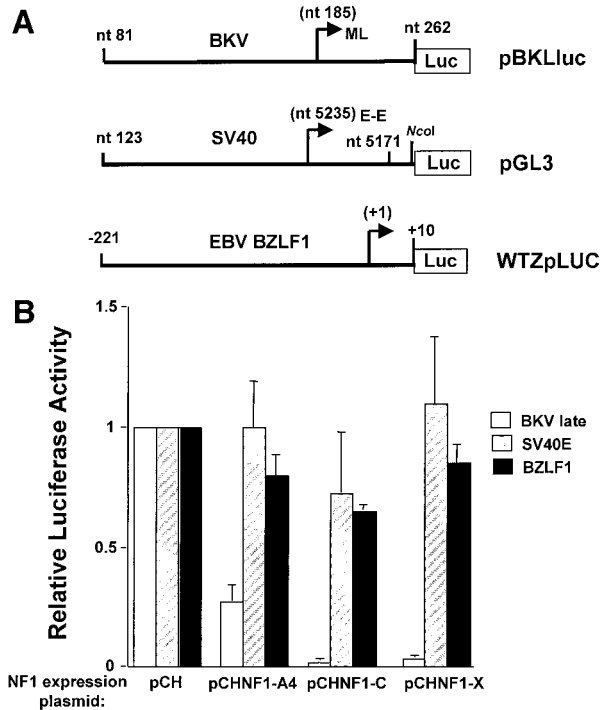


FIG. 9. NF1-A4, NF1-C2, and NF1-X2 repress transcription from the late promoter of BK virus. (A) Schematics of the luciferase reporter plasmids assayed here for effects on expression by overexpressed NF1 family members. The BKV nucleotides are in the numbering system of Negrini *et al.* (1991). The SV40 nucleotides are in the numbering system of Buchman *et al.* (1982). The EBV nucleotides are numbered relative to the transcriptional initiation site of the BZLF1 promoter (Kraus *et al.*, 2001, and references cited therein). (B) Effects of overexpression of NF1-A4, NF1-C2, and NF1-X2 on transcription from the BKV(WW) late, SV40 early, and the EBV BZLF1 promoters. 293 cells grown in 12-well tissue culture dishes were cotransfected with the indicated reporter plasmids (1.0 μ g) and NF1 expression plasmids (0.5 μ g), incubated for 24 h at 37°C, harvested, and assayed for luciferase activity. Luciferase activities were normalized to protein levels for each individual cell extract. They are presented here relative to the activity obtained from cells cotransfected in parallel with the parental expression plasmid, pCH, and the same reporter. These numbers are means \pm SEM of data obtained from three separate experiments, each performed in triplicate.

other groups who showed that the BKV(WW) strain grows quite poorly in culture, probably because of an inactive promoter-enhancer region (Rubinstein and Harley, 1989; Sugimoto *et al.*, 1989; Markowitz *et al.*, 1990). We also failed to detect synthesis of BKV late RNA in our cell-free transcription system when BKV(WW) DNA was used as template (data not shown). Thus, neither an *in vivo* nor an *in vitro* system was available to test directly our hypothesis with the BKV(WW) strain.

Chakraborty and Das (1991) have reported the detection of BKV late RNA in a cell-free transcription system. However, their experiment differed from ours in several respects: (i) they studied the BKV Gardner strain; (ii) they used linearized DNA as template and an unusually high template-to-protein ratio in their reactions; and (iii) their

major late start site mapped approximately 100-bp downstream of the *in vivo* start site.

To overcome the transcriptional inefficiencies of the BKV(WW) promoter, we constructed the hybrid plasmid pSVBK2 (Fig. 2A). Late RNA was readily detectable in COS-M6 cells transfected with this chimeric virus (Fig. 2B), thereby allowing for the first identification of the major site of transcription initiation from the late promoter of this strain (Fig. 2C; summarized in Fig. 2D). We found that the start site lies within the core motif, TGGN₆GGCA, of the nuclear factor 1 (NF1) family of transcription factors and that members of the NF1 family of transcription factors bind to this site (Fig. 3). We demonstrated that the binding of *trans*-acting factors to this site sequence-specifically represses transcription in a concentration-dependent manner both *in vivo* (Fig. 5) and *in vitro* (Fig. 6). Finally, we identified several specific members of the NF1 family, NF1A4, NF1-C2, and NF1-X2, that sequence-specifically bind to the BKV-MLP (Figs. 7 and 8); we showed that these factors can mediate repression of this promoter in a DNA binding-dependent manner (Fig. 9).

We focused on the regulatory role of the NF1 binding site that overlaps the *Inr* region of the BKV-MLP even though the BKV(WW) late promoter contains multiple NF1-binding motifs (Markowitz and Dynan, 1988; Figs. 1 and 10). We postulated that this binding site might function as a negative regulatory element of BKV's late promoter by a mechanism analogous to the way the HRE overlapping the *Inr* of the SV40-MLP does (Wiley *et al.*, 1993). Because the BKV(WW) strain grows so poorly in tissue culture, we were unable to test directly our hypothesis for the temporal regulation of expression of the BKV genes. Nevertheless, we propose the following model (Fig. 10). Following infection of the host, BKV can establish a latent state, most likely in an episomal form (Heritage *et al.*, 1981), in which the early genes are expressed at low levels and expression of the late genes is repressed by cellular factors such as NF1 (Fig. 10A). Under appropriate physiological or exogenous stimuli, the BKV early promoter is activated for a high level of expression, while the late genes remain quiescent (Fig. 10B). When the early gene product, large T antigen, has accumulated to sufficient levels, it binds to the viral origin of DNA replication (*ori*) region, both repressing transcription of the early promoter and initiating replication and amplification of the viral genome (Cassill and Subramani, 1988). Once the viral genome has been amplified to sufficiently high levels, repression of the late promoter is relieved by titration of the repressors, thereby allowing efficient transcription from the promoter, synthesis of the capsid proteins, and packaging of viral genomes into virions (Fig. 10C).

In support of this hypothesis, we found that the *Inr* of the BKV-MLP sequence-specifically binds NF1 family members, repressing transcription, but only when tem-

State after infection

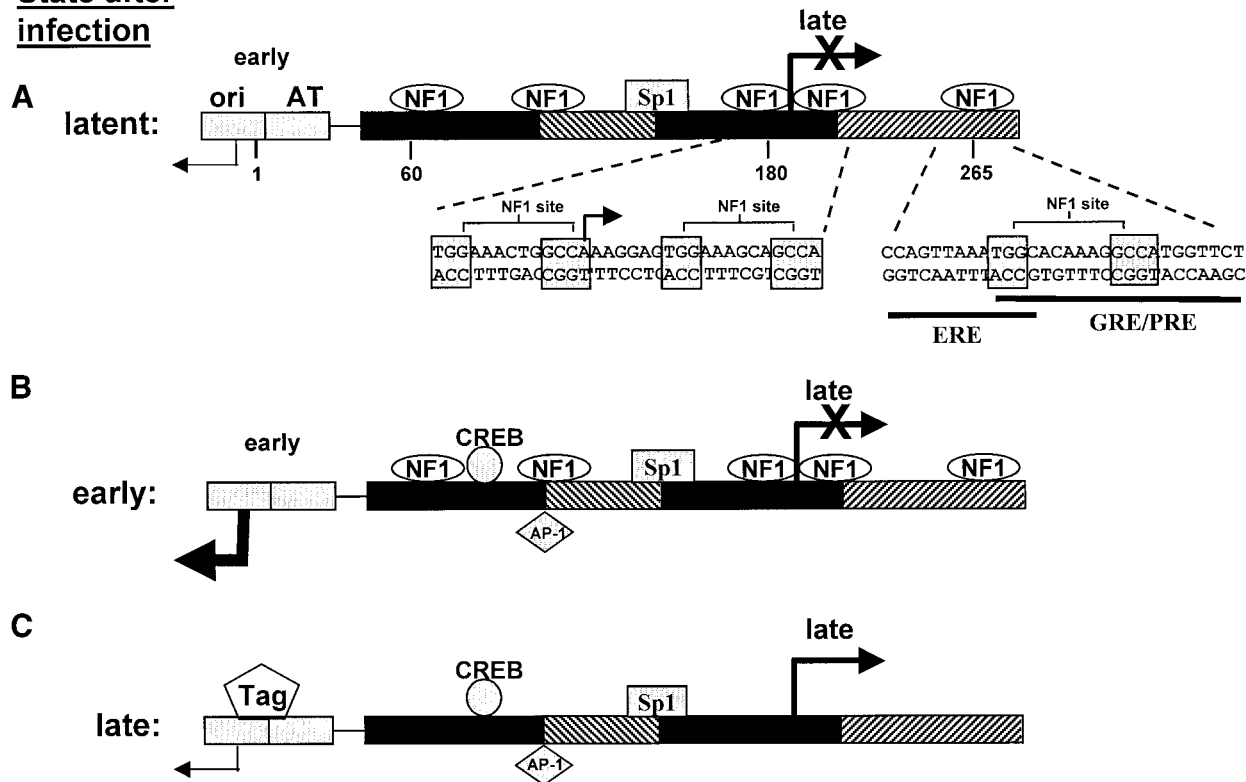


FIG. 10. Model for the mechanism of the replication-dependent component of the early-to-late switch in BKV gene expression. See text for details. Symbols are as shown in Figs. 1 and 3D. The underlined sequences indicate a putative estrogen response element (ERE) and glucocorticoid/progesterone response element (GRE/PRE) (Moens *et al.*, 1994, 1999). Not depicted are other, as-yet-unidentified, factors that may contribute to regulation of transcription from this promoter.

plate copy number is low (Figs. 5 and 6). Interestingly, we failed to observe NF1 DNA-binding activity in nuclear extract obtained from 293 human kidney cells (Fig. 3A), a presumed host cell type. Grinnell *et al.* (1988) reported similar observations. We have detected weak NF1/DNA complexes by EMSAs with 100 μ g of whole-cell extract obtained from 293 cells (data not shown). Thus, while not completely devoid of NF1 DNA-binding activity, 293 cells have, surprisingly, a relatively low abundance of it. Possibly, NF1 is as abundant in 293 cells as it is in HeLa cells, but exists in a complexed or posttranslationally modified state (e.g., phosphorylation) that interferes with DNA binding under the reaction conditions used here. Whether the NF1-binding site that overlaps the Inr acts alone or in combination with other NF1-binding sites present in the promoter remains to be determined.

Some exogenous agents that activate BKV early transcription are known. Moens *et al.* (1990) found agents that stimulate the adenylate cyclase pathway, enhancing BKV early gene expression several fold. These agents signal through a c-AMP responsive element, CREB (Montminy *et al.*, 1986 and references cited therein), located between nts 75 and 83 (Cassill *et al.*, 1989; Moens *et al.*, 1990; Fig. 10B). These same authors also showed that phorbol esters can activate early gene ex-

pression, likely through a putative responsive element located between nts 97 and 104 (Moens *et al.*, 1990).

Regulation by NF1 proteins

NF1 proteins are encoded by a family of four genes, NF1-A, NF1-B, NF1-C, and NF1-X (Chosdosh *et al.*, 1988; Gil *et al.*, 1988; Goyal *et al.*, 1990; Kruse and Sippel, 1994). In addition, these four genes generate isoforms, the result of multiple alternative RNA splicing events. There is considerable sequence homology among these proteins within their amino-terminal ends, which contain the DNA-binding domains. The carboxyl-terminal sequences are quite diverse. Usually, NF1 factors act as transcriptional activators (Miksicek *et al.*, 1987; Santoro *et al.*, 1988; Gronostajski *et al.*, 1988; Chakraborty and Das, 1989; Knox *et al.*, 1991; Chakraborty and Das, 1991). However, there are also multiple examples in which NF1 factors function as transcriptional repressors (e.g., Adams *et al.*, 1995; Osada *et al.*, 1997; Chaudhry *et al.*, 1999). We showed here that NF1-A4, NF1-C2, and NF1-X2 can repress transcription from the BKV late promoter in 293 cells by a sequence-specific mechanism (Fig. 9).

Grinnell *et al.* (1988) found that a HeLa cell-specific

factor(s) bound to NF1 sites present in the BK virus P2 enhancer, repressing transcriptional activity from this enhancer. This factor(s), termed BEF-1 for BK virus enhancer factor 1, is a 98-kDa protein that is possibly related to the NF1 family of proteins (Reifel-Miller *et al.*, 1991). BEF-1 recognizes the NF1 binding site present in the adenovirus origin of replication (Reifel-Miller *et al.*, 1991), the same sequence called NF1 in Fig. 3D. Since the NF1 factors present in HeLa cells that bound to the *Inr* region of BKV(WW)-MLP also bound to the known NF1 site (Fig. 3B), it is quite likely that BEF-1 recognizes the NF1 site overlapping the *Inr* of the BKV(WW)-MLP.

Variants of BKV

The various strains of BK virus are distinguishable by the heterogeneity in their noncoding regions (Fig. 1). Despite complex sequence rearrangements in the enhancer region, the early promoter region and early RNA start sites are conserved among all of the strains of BKV isolated to date (Deyerle *et al.*, 1987; Deyerle and Subramani, 1988, 1989; Markowitz *et al.*, 1990; see Fig. 1). However, considerable heterogeneity exists regarding their late transcriptional start sites.

Cassill and Subramani (1988) mapped a cluster of 5' ends of the late RNAs of the BKV(Gar) strain to a location within the c element situated immediately downstream of the repeat elements (Fig. 1B). The major late transcription initiation site mapped to the sequence 5'-CAGT-3', an optimal *Inr* (Kraus *et al.*, 1996). This *Inr* region also contains an overlapping NF1-binding site. Thus, while the regulatory regions of these two strains are quite different in overall structure, the mechanism of repression of their late promoter may be similar.

Regulation of JC virus

Another human polyomavirus, the JC virus (Grinnell *et al.*, 1983), possesses many features similar to those of BK virus. The archetype, CY (Yogo *et al.*, 1990), has been isolated from human urine and kidneys, while naturally occurring variants with rearranged promoter-regulatory regions have been isolated from human brain tissue (Rencic *et al.*, 1996). Interestingly, all of the JC virus strains contain multiple NF1-binding sites within their promoter-regulatory regions (Amemiya *et al.*, 1992; Raj and Khalili, 1995). Daniel and Frisque (1993) mapped the major late RNA start sites of the JCV(Mad1) strain to nts 191–192 and 200–203. Immediately downstream of these sites (nts 213–219) lies the sequence TGGN₆GCC. Sequence-specific cellular factors that recognize this region have been shown to exist in HeLa and brain nuclear extract (Khalili *et al.*, 1988). Whether some of these NF1-binding sites play roles in the early-to-late switch in expression of JCV has yet to be determined.

In summary, we have shown here that expression of the late genes of BKV can be regulated, in part, by the

sequence-specific binding of at least some members of the NF1 family of transcription factors. Yet to be determined is the regulatory role of the other NF1-binding sites present in this promoter and the molecular mechanism of repression of the BKV-MLP by NF1 factors. We speculate that the binding of repressors to late promoters may be a mechanism frequently employed by DNA viruses to regulate their genes.

MATERIALS AND METHODS

Plasmid DNAs

All plasmids were constructed by standard recombinant DNA techniques (Sambrook *et al.*, 1989). Plasmid pSVS(WT) consists of SV40 wild-type (WT) strain 776 cloned into a pBR322-based vector via their *EcoRI* sites (Buchman *et al.*, 1982; Fromm and Berg, 1982). Plasmid pSV1773(WT) is a derivative of pSVS(WT) containing a frameshift mutation in the VP1-coding region (Good *et al.*, 1988); it was used in transfection experiments to ensure that virion production and, consequently, second cycles of infection did not occur. Plasmid pSV4503(+1/+3GGG) is a derivative of pSV1773(WT) in which SV40's major late promoter (SV40-MLP) is inactive because of a substitution mutation in the initiator element (*Inr*) (Kraus *et al.*, 1996). Plasmid pBKV(WW), containing BKV wild-type strain WW, was obtained from Dr. R.-B. Markowitz.

The SV40-BKV chimeric plasmid pSVBK2 containing the *Bsu36I*-*KpnI* region of pBKV(WW) cloned into the *SphI*-*KpnI* restriction sites of pSV4503 was synthesized as follows. First, plasmid pBKV(WW') was generated by subcloning of the 5701-bp *Bg/II* BKV-containing fragment of pBKV(WW) into pSP72 (Promega Corp.). Subsequently, the SV40-BKV chimeric plasmid pSVBK1 was constructed by substitution of the smaller 1702-bp *SphI*-*KpnI* promoter-containing fragment of pBKV(WW') for the corresponding fragment of pSV4503. Finally, the SV40-BKV chimeric plasmid pSVBK2 was generated by cleavage of pSVBK1 with *SphI* and *Bsu36I*, filling in the single-stranded ends, and relegation (see Fig. 2A).

Plasmid pSV1790 is a derivative of pSV1773 in which SV40 nucleotide residues (nt) 319–336 in the numbering system of Buchman *et al.* (1982) (which include the *Inr* of the SV40-MLP) have been replaced by a sequence containing two *BspMI* sites. Cleavage of pSV1790 with *BspMI* yields a vector containing noncomplementary single-stranded ends into which synthetic oligonucleotides can be ligated to substitute for SV40 nt 319–336 inclusive (Wiley *et al.*, 1993; Kraus *et al.*, 1996). Plasmids pSVBK3 and pSVBK3mut were constructed by ligation into *BspMI*-digested pSV1790 of appropriate double-stranded versions of the synthetic oligonucleotides containing the sequence 5'-TTCAGTGGAACTGGCCAAAGGAG-3' (which includes sequence corresponding to BKV nt –12 through +7 relative to the initiation site of the BKV-MLP) and 5'-TTCAGTTGAACTGGCAAAGGAG-3' (a mutant vari-

ant of it), respectively (see Fig. 4A). Plasmid pXS13 is a derivative pSVS lacking the 72-bp enhancer region of SV40 (i.e., SV40 nt 115–272, inclusive) (Fromm and Berg, 1982). Transcription from the late promoter of this plasmid is activated in a cell-free system via the retained Sp1-binding sites (Wiley *et al.*, 1993; Kraus *et al.*, 1996). The plasmids pSVBK3X and pSVBK3Xmut, used in the cell-free transcription experiments (Fig. 6), were generated by substitution of the promoter-containing *KpnI*–*EcoRV* regions of plasmids pSVBK3 and pSVBK3mut, respectively, for the corresponding region of pXS13.

Plasmid pGL3 (Promega Corp.) contains the SV40 early promoter driving a luciferase reporter (Fig. 9A). Plasmid pBKLluc contains a luciferase reporter driven by the BKV(WW) late promoter (Fig. 9A). It was constructed by cloning the 276-bp *NcoI* restriction fragment of pSVBK2 (see Fig. 2A) into the *NcoI* site of plasmid pGL3. Plasmid pWTZpLUC contains a luciferase reporter driven by the BZLF1 promoter of Epstein–Barr virus (Kraus *et al.*, 2001). The NF1 expression plasmids, pCHNF1-A, pCHNF1-C, and pCHNF1-X, a kind gift from Dr. Richard M. Gronostajski, express HA-epitope-tagged murine proteins that are homologous to chicken NF1-A4, chicken NF1-C2, and human NF1-X2, respectively (Kruse and Sippel, 1994).

All synthetic oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). The sequences of all plasmids were confirmed by DNA sequence analysis.

Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed as described previously (Cooney *et al.*, 1992; Zuo, 1995; Zuo *et al.*, 1997) with nuclear extracts prepared from HeLa cells (Wiley *et al.*, 1993) and CV-1 cells (Zuo, 1995). Nuclear extracts of 293 cells were obtained as described for CV-1 cells. Whole-cell extracts containing overexpressed NF1 proteins were used to assess the binding affinity of specific NF1 proteins for NF1 binding sites present in the BKV late promoter (Figs. 7 and 8). These latter extracts were prepared from five 100-mm dishes of 293 cells, grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS that had been transfected with 6 μ g/dish of the desired NF1 expression plasmid by the Mirus TansIT-LT1 technique (Pan Vera Corp., Madison, WI) (see below). Forty-eight hours posttransfection, the cells were washed with cold PBS, scraped into 2 ml of cold PBS, pelleted, resuspended in 100 μ l of lysis buffer [50 mM Tris–HCl (pH 7.5), 0.5 M NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, the protease inhibitors 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 2.5 μ g/ml pepstatin, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)] and subjected to three freeze–thaw cycles. Cellular debris was removed by centrifugation using a desktop centrifuge. The supernatant was stored at -70°C .

Competition EMSAs were performed by incubation of the reaction mixture with the unlabeled competitor oligonucleotide prior to the addition of the radiolabeled probe DNA. Immunoshift assays were performed by preincubation of the nuclear extract with the indicated sera (generously provided by N. Tanese; Nehls *et al.*, 1991) prior to addition of the probe DNA. All synthetic oligonucleotides serving as probes were gel purified before use.

Cell-free transcription assays

Cell-free transcription assays were performed as previously described (Wiley *et al.*, 1992, 1993). Reaction mixtures (50 μ l) contained 200–250 μ g protein from a HeLa cell nuclear extract and the indicated amounts of supercoiled plasmid DNA. The quantities and the 5' ends of the resulting RNAs were determined by primer extension analysis as described previously (Good *et al.*, 1988) with 5' end-labeled synthetic oligonucleotides corresponding to SV40 nucleotides 446–422 and 5178–5201 serving as primers for the detection of the late and early RNAs, respectively. The effects of competitor oligonucleotides on transcription were determined by incubation of nuclear extract with the competitor oligonucleotide for 15 min at $25\text{--}30^{\circ}\text{C}$ prior to the addition of the template DNA and NTPs. The relative amounts of the various RNAs were quantified by analysis with a PhosphorImager (Molecular Dynamics).

Transient transfection assays

The African green monkey kidney cell lines CV-1PD and COS-M6 were grown in 100-mm dishes in DMEM supplemented with 5 and 10% fetal bovine serum, respectively. All transient transfections were performed by the DEAE–dextran method followed by chloroquine treatment as described previously (Good *et al.*, 1988).

To map the location of the BKV-MLP (Fig. 2), the viral sequences of pSVBK2 were excised from the vector sequences, ligated to form monomer circles, and transfected into COS-M6 cells. Whole-cell RNA was harvested 48 h after transfection. The 5' ends of the pSVBK2 late RNAs synthesized from the chimeric promoter were mapped by primer extension analysis as described previously (Wiley *et al.*, 1992, 1993; Kraus *et al.*, 1996) with the synthetic oligonucleotide 5'-CCGTCTAACACTGTCTTCACCTCT-3', corresponding to BKV nt 227–203 (relative to BKV's major late initiation site), serving as primer.

In the time-course experiment (Fig. 5), the viral sequences of plasmids pSVBK3 and pSVBK3mut were excised from the vector sequences and ligated to form monomer circles prior to transfection. Whole-cell RNA was harvested at the various times indicated. The relative amounts of viral late RNA accumulated in the transfected CV-1PD cells were determined by quantitative S1 nuclease mapping as previously described (Hertz and Mertz, 1988). The S1 nuclease mapping probe was pre-

pared by PCR with synthetic oligonucleotides corresponding to SV40 nt 4924–4949 and 446–422 serving as primers and plasmid pSVBK3(WT) serving as template (Fig. 5C). Southern blots were performed as described previously (Hertz and Mertz, 1986).

To assess the role specific NF1 factors play in regulating transcription of the BKV promoter (Fig. 9), 0.5 μg of the indicated NF1 expression plasmids were cotransfected along with 1.0 μg of the desired luciferase reporter plasmid into 293 cells in 12-well tissue culture dishes, grown as described above, with the aid of the Mirus TransIT LT1 transfection reagent (Pan Vera). Briefly, 4 μl of TransIT LT1 reagent was incubated at room temperature for 10 min with 100 μl of serum-free DMEM. Next, 1.5 μg of total DNA was added to this mixture and incubated for an additional 10 min. The TransIT LT1/DNA complex mixture was added dropwise to cells growing in 1 ml of fresh growth media. Incubation was continued and cells harvested at 24 h posttransfection.

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